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COMPLETION OF THE LIFE CYCLE OF *SARCOCYSTIS NEURONA*

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ABSTRACT: *Sarcocystis neurona* is the most important cause of a neurologic disease in horses, equine protozoal myeloencephalitis (EPM). The complete life cycle of *S. neurona*, including the description of sarcocysts and intermediate hosts, has not been completed until now. Opossums (*Didelphis* spp.) are definitive hosts, and horses and other mammals are aberrant hosts. In the present study, laboratory-raised domestic cats (*Felis domesticus*) were fed sporocysts from the intestine of a naturally infected opossum (*Didelphis virginiana*). Microscopic sarcocysts, with a maximum size of 700 × 50 μm, developed in the muscles of the cats. The DNA of bradyzoites released from sarcocysts was confirmed as *S. neurona*. Laboratory-raised opossums (*D. virginiana*) fed cat muscles containing the sarcocysts shed sporocysts in their feces. The sporocysts were ~10–12 × 6.5–8.0 μm in size. Gamma interferon knockout mice fed sporocysts from experimentally infected opossums developed clinical sarcocystosis, and *S. neurona* was identified in their tissues using *S. neurona*-specific polyclonal rabbit serum. Two seronegative ponies fed sporocysts from an experimentally-infected opossum developed *S. neurona*-specific antibodies within 14 days.

Sarcocystis neurona is an unusual species of *Sarcocystis*. It is the most important cause of a neurological disease of the horse (equine protozoal myeloencephalitis, EPM), and EPM is the most commonly diagnosed neurologic disease of the horse in the United States (Dubey et al., 1991; Dubey, Lindsay, Saville et al., 2001). EPM causes annual loss of more than 100 million dollars to the equine industry in the United States (Dubey, Lindsay, Saville et al., 2001). *Sarcocystis neurona*–associated EPM-like disease also occurs in other mammals in North America, including sea otters, Pacific harbor seals, skunks, mink, raccoons, cats, and ponies (reviewed by Dubey, Lindsay, Saville et al., 2001). The life cycle of *S. neurona* is incompletely known. Opossums (*Didelphis* spp.) are definitive hosts that excrete sporocysts in feces (Fenger at al., 1997; Dubey and Lindsay, 1998; Dubey, Lindsay, Kerber et al., 2001). How opossums become infected with *S. neurona* is unknown because neither the sarcocyst stage nor the intermediate host that harbors the sarcocyst stage are known. The horse is considered an aberrant host because only schizonts are known, and they are confined to the central nervous system. Progress on the epidemiology and control of EPM is hampered because the life cycle has not been completed in the laboratory and opossums are hosts to more than 3 species of *Sarcocystis*, whose sporocysts cannot be distinguished morphologically with certainty (Box and Smith, 1982; Dubey and Lindsay, 1999; Tanhauser et al., 1999).

In the present study, we have identified the sarcocyst stage of *S. neurona* and completed the life cycle in the laboratory using domestic cats (*Felis domesticus*) as experimental intermediate hosts. Cats were fed *S. neurona* sporocysts from feces of a naturally infected opossum, and muscles from cats were fed to laboratory-raised opossums that later excreted *S. neurona* sporocysts. Details of sarcocyst morphology, pathology, and biology of *S. neurona* in cats inoculated by different routes and with various stages of *S. neurona* will be addressed later.

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MATERIALS AND METHODS

Sarcocystis neurona sporocysts

Sporocysts of the SN15-OP isolate were obtained from intestinal scrapings of a naturally infected opossum (no. 9108) as described by Dubey (2000). They were stored in antibiotics (Leek and Fayer, 1979) at 4 C for 177 or 292 days before feeding to cats. The inoculum contained *S. falcatula* and *S. neurona*, but not *S. speeri* as confirmed by bioassay (Dubey, 2000). The proportion of *S. falcatula* versus *S. neurona* sporocysts was unknown (Dubey, 2000). However, by bioassay in gamma interferon knockout (KO) mice, ~100,000 *S. neurona* were present in 1 ml of the undiluted suspension (Dubey, 2001).

Inoculation of cats

Laboratory-raised cats from a parasite-free colony were used. The management of this cat colony has been reported (Dubey, 1995). The cats were 5–7-mo old at the time of sporocyst feeding (Table I). Two experiments were performed. For feeding suspensions of sporocysts, the cat's head was held in an upward position and 2.5 ml (cats nos. 476, 491, experiment 1) or 1 ml (cats nos. 511, 525, 536, experiment 2) of inoculum was poured in the mouth. Three cats were given cortisone (methyl prednisolone acetate [Pharmacia & Upjohn Co, Kalamazoo, Michigan], 40 mg/kg body weight) as shown in Table I. Cats were housed individually, were given food and water ad lib., and were observed up to 144 days (Table I). Feces from each cat were collected daily and incinerated to avoid cross contamination. Five cats were killed 36, 45, 57, and 144 days after feeding sporocysts (Table I). One uninoculated control cat (no. 543), housed in the room where inoculated cats were housed, was killed on day 144 of the experiment (Table I).

Examination for sarcocysts

Cats were necropsied on the days indicated in Table I. Muscles from tongue, heart, diaphragm, abdomen, and the rest of the carcass were examined for sarcocysts by squash preparation between coverslips and slides, by histology, by electron microscopy, and by DNA analysis by polymerase chain reaction (PCR). For histological examination, muscles were fixed in 10% neutral buffered formalin (NBF), sectioned at 5μm, and stained with hematoxylin and eosin (H and E). Formalin-fixed muscles from cat no. 476 (experiment 1) were postfixed in osmium and processed for transmission electron microscopic examination as described (Speer et al., 1997), except they were prestained with 1% (w/

TABLE I. *Sarcocystis neurona* infection in cats fed sporocysts from a naturally infected opossum.

Exp. no.	Cat no.	Days old	Sporocysts fed	Cortisone given	Day of necropsy	Sarcocysts in muscle	Muscles fed to opossums	Opossum no.	Sporocysts in opossums
1	476	152	Yes	Yes*	45	Immature	No	NA‡	NA
	491	140	Yes	No	57	Immature	No	NA	NA
2	511	166	Yes	Yes†	36	Immature	No	NA	NA
	525	142	Yes	Yes†	144	Mature	Yes	5, 6§	16×10^6, 6×10^6
	536	142	Yes	No	144	None	Yes	3, 4§	Few#
	543	163‖	No	No	144	None	Yes	1, 2§	None

* 40 mg/kg at 0, 14, and 35 days after feeding sporocysts.
† 40 mg/kg on day 7 before feeding sporocysts and on days 7 and 20 after feeding sporocysts.
‡ NA, not applicable.
§ Littermates.
‖ Age on the day of necropsy.
<1,000.

v) phosphotungstic acid and 1% (w/v) uranyl acetate in 70% ethanol. Ultrathin sections were stained with lead citrate and examined with a JEOL 100CX electron microscope.

To release bradyzoites from sarcocysts, 100 g of muscle from legs of cats in experiment 2 was homogenized, digested in acid pepsin, and washed, and the organisms were separated on Percoll as described (Dubey et al., 1989).

Opossums

Pregnant wild opossums (*D. virginiana*), trapped in Florida, were received at the Ohio State University (OSU) research facility. They were given water and dry dog food ad lib., as well as orange sections. Twenty-seven days following arrival (dfa) at OSU, the first kits from opossum 1 were observed out of the pouch. There were 6 kits in this litter and on 55 dfa, 3 of the kits from this litter were weaned and placed in separate rat cages. On 56 dfa, the other 3 kits were weaned and placed in separate cages.

All opossum kits were individually housed in rat cages with wood chips for bedding and fed dry dog food and water ad lib., along with bananas. Cages were cleaned daily. One day prior to the start of the study, all kits were fasted for 12 hr. On day 0, fecal samples were collected from all kits to examine for evidence of sporocysts, and fecal samples were collected daily thereafter. Sugar flotations were performed according to previously published methods (Dubey et al., 1989). Two kits were fed muscles from each cat (Table I). Each kit was given cat muscle (~250 g), along with small amounts of dry dog food. This feeding schedule was continued until all of the meat had been consumed. On day 14 after feeding cat muscles, opossums were killed with CO_2 and their small intestine was removed after it was tied at the pylorus and ileum. The intestine sections were shipped overnight on ice to the USDA laboratory. Sporocysts were collected from intestinal scrapings, counted with a hemacytometer, and stored in antibiotics at 4 C as described (Dubey, 2000).

TABLE II. Results of immunohistochemical examination of tissues of KO mice fed *Sarcocystis neurona* sporocysts from opossum nos. 5 and 6 fed cat muscles.

Opossum no.	KO mouse no.	Day killed/ died*	Tissues positive for *S. neurona*†	*S. neurona* agglutination test‡
5	7,152	K2	I, Ml	ND
	9,784	K6	H, Ml, Li, Lu, Sk, Sp	ND
	9,785	K8	B, H, Li, Lu, Ty	ND
	9,783	K10	B, H, Li, Lu, Sk, Te, To	1:50
	9,782	K12	B, Li, Lu, Sp, To	1:50
	9,800	D11	B, H, Li, Sk	1:50
	9,799	Dk16	B, H, Lu	>1:500
	9,802	Dk19	B, H, Lu	>1:500
	9,801	Dk21	B	>1:500
	9,803	Dk21	B	>1:500
6	9,814	Dk20	B, H, Lu	>1:500
	9,815	Dk20	B, Lu	>1:500

* D, died; K, killed; Dk, killed when ill.
† B, brain; H, heart; I, intestine; Ml, mesenteric lymph nodes; Li, liver; Lu, lung; Sk, skeletal muscle; Sp, spleen; Te, testes; To, tongue; Ty, thymus.
‡ ND, not done.

Bioassay for *S. neurona* in mice

Sporocysts from intestinal scrapings of opossums fed cat muscles were bioassayed in gamma interferon KO mice as described by Dubey and Lindsay (1998). Twelve 6-wk-old KO mice were each fed ~250,000 sporocysts by stomach tube. Five KO mice were killed on days 2, 6, 8, 10, and 12 after receiving sporocysts as planned, and the remaining mice were killed when they became ill (Table II). All tissues from mice killed or that died were fixed in NBF and processed for immunohistochemical examination as described (Dubey et al., 1999; Dubey and Hamir, 2000). A 1:5,000 dilution of polyclonal anti-*S. neurona* serum from a rabbit immunized with the SN12-OP isolate (Dubey et al., 1999) was used as described (Dubey and Hamir, 2000) for immunohistochemistry.

Attempted infection of budgerigars

Sporocysts (~250,000) from opossum nos. 5 and 6 were fed to 4 budgerigars (*Melopsittacus undulatus*) as described (Dubey and Lindsay, 1998). The budgerigars were killed 14 (2 fed sporocysts from opossum 5) or 33 (2 fed sporocysts from opossum 6) days later and their tissues were fixed in NBF, sectioned at 5 μm, stained with H and E, and examined microscopically for *Sarcocystis* stages.

Serologic examination for *S. neurona* antibodies

Sera from mice were examined for antibodies to *S. neurona* using the *Sarcocystis neurona* agglutination test as described by Lindsay and Dubey (2001).

In vitro cultivation of *S. neurona* from cat brain

Brain homogenate from cat no. 511 (killed 36 days after feeding sporocysts) was inoculated onto bovine turbinate cells (BT cells, ATCC CRL 1390, American Type Culture Collection, Rockville, Maryland) as described (Dubey et al., 1999; Lindsay et al., 1999).

Molecular characterization

Polymerase chain reaction (PCR) was performed on merozoites of *S. neurona* (isolate SN6 and those isolated from cat no. 511 of the present study), *S. falcatula* (merozoites from the Cornell isolate), and bradyzoites from cat no. 525 using methods described previously (Dubey, Lindsay, Venturini, and Venturini, 2000). The *S. neurona* and *S. falcatula* primers (JNB33 and JNB54) developed by Tanhauser et al.

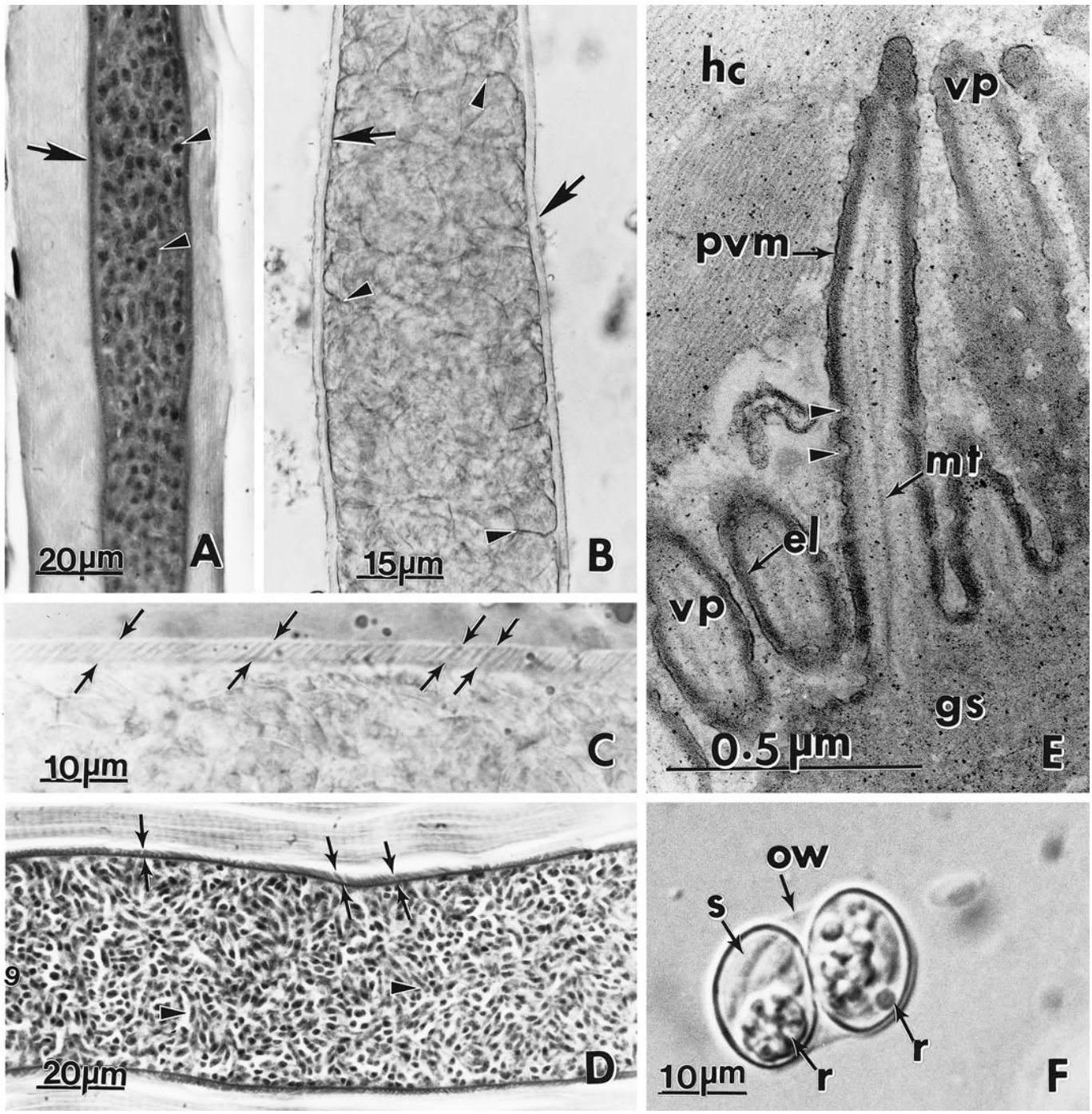

FIGURE 1. Stages of *Sarcocystis neurona*. **A.** Immature sarcocyst in skeletal muscle of cat no. 511, 37 days after feeding sporocysts (DAFS). Hematoxylin and eosin stain. Note metrocytes (arrowheads) and prominent cyst wall (arrow). **B.** Sarcocyst in skeletal muscle of cat no. 525, 144 DAFS. Note cyst wall (arrows) and septa (arrowheads). Unstained. **C.** Higher magnification of cyst wall showing slender villar protrusions (arrows) on cyst wall. Unstained. **D.** Sarcocyst in longitudinal section. Note cyst wall with villar protrusions (arrows) and small, slender bradyzoites (arrowheads). Hematoxylin and eosin stain. **E.** Transmission electron micrograph of cyst wall of a sarcocyst from cat no. 476, 45 DAFS. Note villar protrusions (vp) with a thin parasitophorous vascular membrane (pvm) lined by an electron-dense layer (el), which is interrupted (arrows) at irregular distances; microtubules (mt) that extend up to the base of the villus; and ground substance (gs) of the cyst wall. **F.** Sporulated oocyst from intestine of opossum no. 5. Note thin oocyst wall (ow), 2 sporocysts, sporozoites (s), and residual bodies (r).

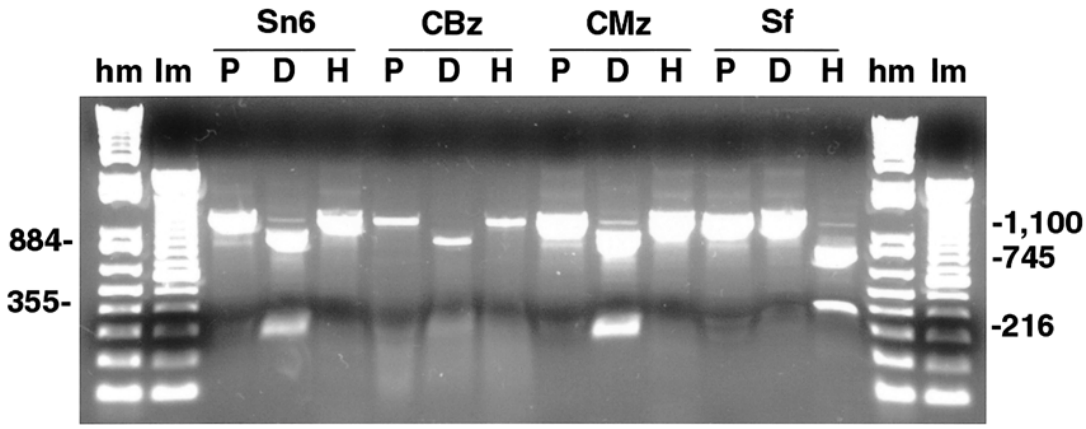

FIGURE 2. Results of PCR using primer pairs JNB33/JNB54 and restriction enzyme digestion with *Dra*I and *Hin*fI of merozoite DNA from *Sarcocystis neurona* isolate SN6 (Sn6), bradyzoites from cat no. 525 (CBz), merozoites from cat no. 511 (CMz), and the merozoites of Cornell isolate of *Sarcocystis falcatula* (Sf); hm, high molecular mass marker; lm, low molecular mass marker; P, PCR product; D, *Dra*I digest; H, *Hin*fI digest.

(1999) were used for PCR reaction, followed by digestion with *Dra*I or *Hin*fI.

Attempts were made to genetically compare merozoites isolated from cat no. 511 and bradyzoites from cat 525 in this study with *S. neurona* described previously from horses (Tanhauser et al., 1999). For this, a DNA template for PCR amplification was prepared from cell culture–derived merozoites from cat no. 511 as previously described by Rosenthal et al. (2001). A locus previously shown to differentiate opossum-derived *Sarcocystis* isolates was amplified via PCR with primers of 25/396 (Tanhauser et al., 1999) and was sequenced in both directions.

Attempted infection of ponies

Sporocysts from opossum no. 5 were administered by nasogastric intubation to 2 seronegative weanling ponies. One pony (B1) received 5×10^4 sporocysts and the other (A1) received 5×10^5 sporocysts.

RESULTS

In cats fed sporocysts from the opossum, sarcocysts were found in muscles of the tongue, diaphragm, abdomen, legs, spine, and head. Sarcocysts found at 36, 45, and 57 days after feeding sporocysts contained only metrocytes, and not all sarcocysts at 144 days were mature. They were microscopic; the longest sarcocyst in section was 700 μm and the widest sarcocyst was 50 μm. The sarcocyst wall appeared relatively thin under the light microscope (Figs. 1A, B). Slender villar protrusions up to 2.5 μm long were visible at the highest magnification (Figs. 1C, D). Ultrastructurally, villar protrusions contained microtubules (Fig. 1E). Bradyzoites were slender, about 5–7 μm long (Fig. 1D).

Two opossums (nos. 5, 6) fed sarcocyst-infected meat from cat no. 525 (given cortisone) shed sporocysts after prepatent periods of 11 and 13 days. A total of 16×10^6 and 6×10^6 sporocysts was recovered from intestinal scrapings of opossums nos. 5 and 6, respectively. The sporocysts were 10.5–12.0 × 6.5–8.0 μm (n = 20) in size. Each sporocyst contained 4 banana-shaped sporozoites and a compact or dispersed residuum (Fig. 1F). Only a few sporocysts were found in intestinal digest of opossums 3 and 4 fed muscles from cat 536 (not given cortisone). Sporocysts were not found in intestinal digest of opossums 1 and 2 fed muscles from control cat 543 (Table I).

Sarcocystis neurona schizonts, merozoites, or sporozoites were found in tissues of KO mice fed sporocysts (Table II). *Sarcocystis neurona* was found mainly in visceral tissues during the first 10 days of infection. Beginning at 8 days postinfection, organisms were seen consistently in the brain.

Merozoites and schizonts were seen in BT cells 27 days after inoculation with brain homogenate from cat no. 511. Schizonts developed as described for *S. neurona* (Lindsay et al., 1999). Merozoites from cat no. 511 and bradyzoites from cat 525 were molecularly identical to previously reported isolates of *S. neurona* also sequenced at the 25/396 locus (Tanhauser et al., 1999). The DNA from bradyzoites from cat no. 525 produced a 1,100-base pairs product that was cut by *Dra*I, but not by *Hin*fI primers at the 33/54 locus (Fig. 2).

The 4 budgerigars fed sporocysts from opossum nos. 5 and 6 remained clinically normal. *Sarcocystis* parasites were not found in tissues of budgerigars fed sporocysts from opossum nos. 5 and 6.

Antibodies were detected by a *S. neurona*-specific immunoblot (Dubey, Lindsay, Saville et al., 2001) in sera of pony B1 on day 7 post-inoculation (PI) and pony A1 on day 14 PI. Neurologic deficits were detected by a masked examiner in pony B1 on day 14 PI.

DISCUSSION

The sarcocyst stage found in the muscles of cats was considered to be *S. neurona* based on antigenic, molecular, and biologic evidence. DNA from bradyzoites from sarcocysts was identified as *S. neurona*. The sporocysts obtained from experimentally infected opossums induced clinical sarcocystosis in KO mice, and the diagnosis was confirmed immunohistochemically. The pattern of tissue parasitization with respect to duration of infection was the same as in KO mice fed sporocysts from feces of the naturally infected opossum that was the source of infection for cats (Dubey, 2001). In addition, KO mice fed sporocysts derived from the cat–opossum cycle developed antibodies to *S. neurona.* These sporocysts from experimentally infected opossums were not infective to budgerigars, indicating they were not *S. falcatula* or related species. Sarcocysts were not found in budgerigars fed sporocysts from opossums nos. 5 and 6, indicating that they were not naturally

infected and immune to *S. falcatula.* Even a few sporocysts of *S. falcatula* are lethal to budgerigars (Box et al., 1982). The 2 budgerigars fed sporocysts from naturally infected opossum no. 9108 that was the source of infection for cats in the present study died of acute sarcocystosis 10 days after feeding sporocysts (Dubey, 2000). Natural infection of cats used in the present study with sarcocysts is not likely because sarcocysts have never been seen in hundreds of cats used by the senior author (J.P.D.) for *Toxoplasma gondii* research.

In the present study, sarcocysts found at 36, 45, and 57 days after feeding sporocysts contained only metrocytes, and not all sarcocysts were mature at 144 days postinfection. These observations suggest a slow rate of maturation for *S. neurona* sarcocysts. Sarcocysts were seen in 1 cat and detected by bioassay in another that were not given prednisolone, indicating that immunosuppression is not necessary for the *S. neurona* cycle to be completed in this experimental intermediate host. Cats were given prednisolone with the objective of inducing *S. neurona*–associated clinical encephalitis because *S. neurona*-like encephalitis was previously diagnosed in a naturally infected cat (Dubey, Higgins et al., 1994; Dubey and Hamir, 2000).

Whether cats are a natural intermediate host of *S. neurona* remains to be determined. Morphologic identification of the sarcocyst stage of *S. neurona* will make it possible to obtain pure *S. neurona* sporocysts for infection of horses and should facilitate the search for its natural intermediate hosts.

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